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Free Radicals Mediate Peroxidative Damage in Guinea Pig Hippocampus In Vitro

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Previous studies have shown that peroxide caused electrophysiological damage. The present study investigates the action of agents that interfere with a free radical process in an effort to define the mechanism of peroxide damage. Deferoxamine chelates iron, making it unavailable for the Fenton reaction and thereby preventing the formation of hydroxyl free radicals from peroxide. Dimethylsulfoxide (DMSO) scavenges hydroxyl free radicals. Trolox-C, a water soluble Vitamin E analog, is an antioxidant that can scavenge peroxy radicals. Slices of hippocampus were removed from brains of euthanized guinea pigs. Electrical stimulation of an orthodromic pathway to CA1 region evoked a synaptic response and a population spike. Input-output curves were generated to evaluate the protection by deferoxamine, Trolox-C, and DMSO on the synaptic damage and impaired spike generation caused by peroxide. Lipid peroxidation was measured by the thiobarbituric acid test. Peroxide was found to increase lipid peroxidation. Deferoxamine and Trolox-C protected against the peroxide-induced synaptic damage, impaired spike generation, and lipid peroxidation. DMSO was ineffective synaptically but reduced peroxide damage to spike generating mechanisms and further lipid peroxidation. The data support the hypothesis that peroxide causes damage through a free radical mechanism.

Key words: hydroxyl free radicals, hydrogen peroxide, lipid peroxidation, deferoxamine, Trolox-C, DMSO

INTRODUCTION

Hydrogen peroxide has been used as a model for free radical damage in a number of systems (Ward et al., 1985; Pellmar, 1986, 1987; Mello Filho et al., 1984; van der Zee et al., 1985). Using the hippocampal brain slice from guinea pigs, previous studies (Pellmar, 1986, 1987) revealed that peroxide decreases the synaptic field potential and the intracellularly recorded excitatory and inhibitory postsynaptic potentials. In addition, peroxide

impairs the ability of the synaptic response to generate an action potential (Pellmar, 1986) and the ability of the cell to produce a train of action potentials (spike frequency potentiation) (Pellmar, 1987).

Hydrogen peroxide reacts with transition metals such as copper and iron to produce the very reactive hydroxyl free radical through the Fenton reaction. It is likely that generation of this oxygen radical is responsible for at least some of the damage caused by peroxide. The hydroxyl radical is a strong oxidant and an initiator of lipid peroxidation. The present study was designed to evaluate the free radical involvement in peroxidative damage to the hippocampus in vitro. The reagents we used were chosen to interfere with different steps in free radical damage: a) deferoxamine to chelate iron and render it inaccessible for the Fenton reaction (Graf et al., 1984); b) DMSO and thiourea to scavenge hydroxyl free radicals (Littlefield et al., 1988; Halliwell and Gutteridge, 1985; Reuvers et al., 1973; Chapman et al., 1973); and c) Trolox-C, an antioxidant, to provide protection, in part, by scavenging the peroxy radical (Doba et al., 1985; Niki, 1987; Halliwell and Gutteridge, 1985; Burton et al., 1985). The effectiveness of these protectants was evaluated on lipid peroxidation and on the electrophysiological deficits produced by hydrogen peroxide in hippocampal slices.

MATERIALS AND METHODS

Slices of hippocampus were prepared from brains of male Hartley guinea pigs as previously described (Pellmar, 1986, 1987). Animals were anesthetized with halothane and euthanized by cervical dislocation. The brain was quickly removed from the animal and chilled in iced artificial cerebrospinal fluid (aCSF) with the fol-

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lowing composition (in mM): NaCl, 124; KCl, 3.0; CaCl_2 , 2.4; MgSO_4 , 1.3; KH_2PO_4 , 1.24; glucose, 10; and NaHCO_3 , 26; equilibrated with 95% O_2 /5% CO_2 . The hippocampus was then dissected bilaterally. Slices 400–450 μm thick were cut on a McIlwain tissue chopper and incubated at room temperature for at least an hour for both electrophysiology and lipid peroxidation experiments.

Deferoxamine (desferal, deferoxamine mesylate) was provided by CIBA Pharmaceutical Company. Trolox-C (Ro20-9747) was a gift from Hoffmann-La Roche, Inc. Thiourea, dimethylsulfoxide (DMSO), trichloroacetic acid, 2-thiobarbituric acid, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co., St. Louis, MO. Malonaldehyde bis(dimethyl acetal) (MDA) was obtained from Aldrich Chemical Co., Milwaukee, WI. Hydrogen peroxide was diluted daily from a 50% stock solution from Fisher Scientific Co., Pittsburgh, PA.

Electrophysiology

A single hippocampal slice was placed in a submerged slice chamber for electrophysiological recordings. Artificial CSF (aCSF) at $30^\circ \pm 1^\circ\text{C}$ and saturated with 95% O_2 /5% CO_2 was continually perfused through the chamber at about 1 ml/min.

A bipolar stainless steel stimulating electrode (DKI) was positioned in the stratum radiatum to stimulate afferents to CA1 region of hippocampus. A glass recording electrode filled with 2 M NaCl was positioned in the stratum pyramidale of CA1 to record the population spike (somatic response). A second recording electrode was placed in the stratum radiatum to record the population postsynaptic potential (dendritic response, population PSP) and the afferent volley, the reflection of the number of afferent fibers firing. The PSP was quantitated by the initial slope of the potential since the evoked population spike frequently prevented measurement of the full amplitude. Constant current stimuli (0.1–1 mA, 200 μs) were applied to the stimulating electrode at 0.2 Hz. The resultant field potentials were recorded with high gain DC amplifiers and were digitized, stored, and analyzed on a PDP 11 computer.

Following placement of electrodes, field potentials were recorded for at least 30 min to ensure a stable preparation. Stimulus intensity was set to a level that produced approximately a half-maximal response and responses (at 0.2 Hz) were continually recorded to monitor the viability of the tissue. Input-output (I/O) curves were generated by varying the stimulus intensity from 0 to 1.0 mA. Curves were obtained first in aCSF. The protectant was then perfused through the chamber. Following a 30 min exposure, another I/O curve was generated. Hydro-

gen peroxide (0.005%) plus the protectant was then applied for another 30 min and a third I/O curve obtained.

The input-output curves consist of three relationships: 1) afferent volley vs. population PSP, 2) population PSP vs. population spike, and 3) afferent volley vs. population spike. A plot of the afferent volley vs. the population PSP reveals any change in the ability to produce a synaptic potential (synaptic damage). A plot of the population PSP vs. the amplitude of the population spike reveals any change in the ability of the synaptic potential to generate an action potential (spike generation damage). The plot of afferent volley vs. population spike reflects the composite of synaptic and postsynaptic damage.

The input-output curves were analyzed as described previously (Tolliver and Pellmar, 1987; Pellmar and Neel, 1989). In short, the response amplitudes recorded at each stimulus intensity were averaged for all experiments under each experimental condition. The average responses and their standard errors were used to construct the curves in Figures 1 and 2. A sigmoid curve was computer fit to the points. Differences between curves with and without peroxide were tested for significance by comparing the residual sum of squares for the individual curves with the residual sum of squares for the curve fit to all the points under both experimental conditions. Significance was accepted at $P < 0.05$.

Lipid Peroxidation

Lipid peroxidation was evaluated through the thiobarbituric acid test as described by Kovachich and Mishra (1980). Slices from one hippocampus were divided into three groups: a) incubated in aCSF for 1 hr b) treated with protectant for 1 hr, and c) treated with protectant for 30 min followed by a 30 min exposure to 0.01% hydrogen peroxide in the presence of the protectant. These 1 hr incubations were done at $30^\circ \pm 1^\circ\text{C}$. At least three slices of hippocampus in each group were necessary to provide sufficient tissue for assay. Each experimental condition was tested at least ten times. After treatment, slices were blotted dry and weighed. Tissue was then homogenized in 1 ml of 20% trichloroacetic acid with 0.5 mM EDTA. Thiobarbituric acid (0.67%) in 20 mM NaOH (2 ml) was added, the solution boiled for 10–15 min and then centrifuged for 10 min at 2,400 rpm. The absorbance was measured at 530 nm. Standards of 0.0 to 15 nmoles of malonaldehyde in 500 μl of aCSF containing 0.5 mM EDTA were treated as was the tissue.

To ensure that the protectants did not influence the measured MDA, standard curves were constructed in the presence of 20 μl of the reagents. This volume was the estimated maximum residual volume of aCSF in the tissue after blotting dry, based on wet weight vs. dry weight measurements for sample slices. Two to three

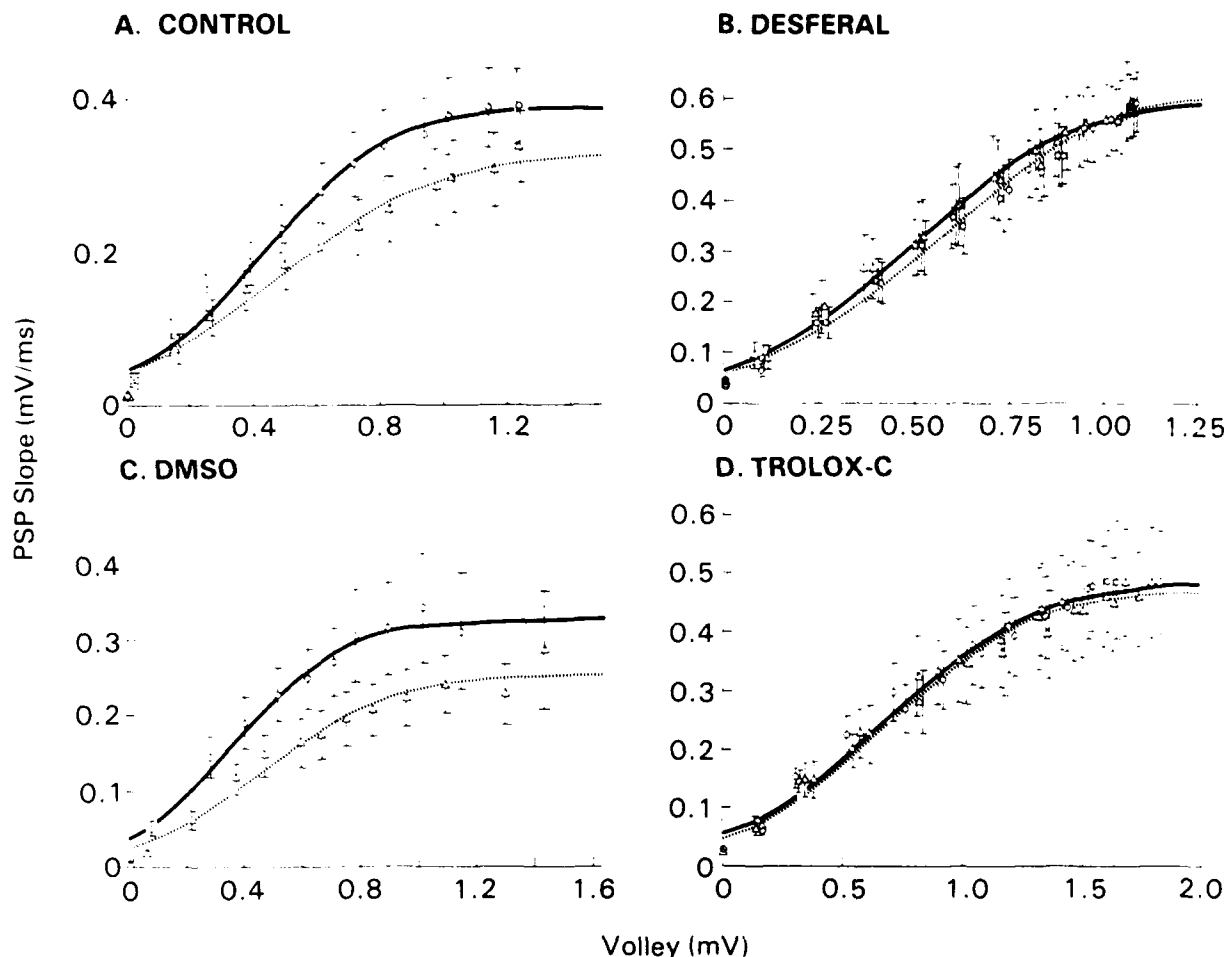


Fig. 1. Input Output curves showing the effect of peroxide (0.005%) on the relationship of the afferent volley and the size of the synaptic potential (A) in the absence of any protectant (Control) and in the presence of (B) deferoxamine (1 mM), (C) DMSO (50 mM) or (D) Trolox-C (100 μ M). Hydrogen peroxide severely decreases the ability of the afferent stimulation to evoke a synaptic potential. Deferoxamine and Trolox-C sig-

nificantly protect against this damage. DMSO does not provide significant protection. None of the three protectants by themselves shifted these curves (not shown). In all graphs, solid lines (circles) show relationship before application of peroxide, with protectant present in B, C, and D. Dotted lines (triangles) show relationship following a 30 min exposure to 0.005% peroxide.

standard curves were constructed for each protectant and for each protectant plus peroxide. There was no significant influence of any of these agents on the standard curves.

RESULTS

Protectants on Electrophysiological Damage

Figure 1 illustrates the effects of deferoxamine, Trolox-C, and DMSO on the damage caused by 0.005% peroxide at the synaptic site. At the doses used these agents had no direct effects on this relationship. In the absence of any protectants (Control), peroxide reduced the ability of orthodromically stimulated afferent fibers

to evoke a synaptic potential in the stratum radiatum. In Figure 1A, data from nine hippocampal slices were combined to produce the curves relating the afferent volley amplitude to the population PSP. A statistically significant change in the curve resulted from exposure to peroxide. In contrast, when peroxide was applied in the presence of 1 mM deferoxamine (desferal) (Fig. 1B), no significant shift in the control curve resulted ($n=5$); deferoxamine protected against peroxide-induced synaptic damage. Similarly Trolox-C (100 μ M), a water soluble analog of vitamin E, was very effective in preventing peroxide-induced synaptic damage ($n=6$) (Fig. 1D). With Trolox-C present, the relationship of afferent volley to synaptic potential was not significantly shifted by per-

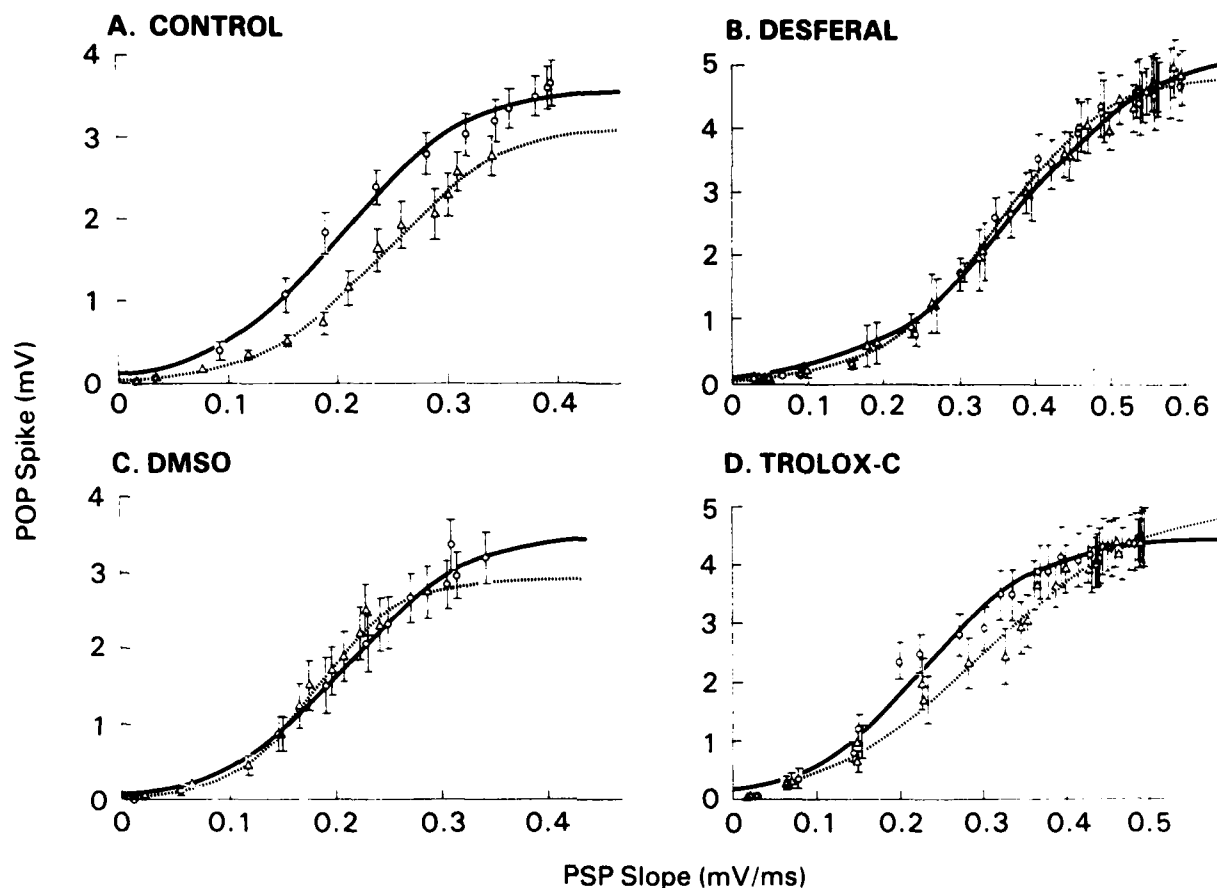


Fig. 2. Input-output curves showing the effect of peroxide (0.005%) on the relationship of the synaptic potential size to the amplitude of the population spike (A) in the absence of any protectant and in the presence of (B) deferoxamine (1 mM), (C) DMSO (50 mM) or (D) Trolox-C (100 μ M). Hydrogen peroxide significantly reduced the ability of a synaptic potential to evoke a spike. All three protectants provided some pro-

tection from this damage. None of the three protectants by themselves shifted these curves (not shown). In all graphs solid lines (circles) show relationship before application of peroxide, with protectant present in B, C and D. Dotted lines (triangles) show relationship following a 30 min exposure to 0.005% peroxide.

oxide. In DMSO (0.36%, 50 mM) (Fig. 1C), however, peroxide produced at least as much synaptic damage as in the absence of any protectants ($n=7$). Despite the presence of DMSO the curve was significantly shifted by addition of peroxide.

In addition to synaptic damage, peroxide has been previously shown to decrease the ability of the synaptic potential to evoke an action potential. This action can be seen in Figure 2A. Addition of peroxide produced a significant shift in the relationship between population PSP size and population spike amplitude. All three protectants afforded some degree of protection against this decrement, with no direct effect of their own on the I/O curve. Deferoxamine ($n=5$) and DMSO ($n=7$) prevented any significant shift in the ability of the synaptic potential to evoke a population spike with exposure to

peroxide. In Trolox-C ($n=6$) a reduction in population spike generation was still present but was substantially reduced compared to peroxide alone.

The ability of Trolox-C to protect against peroxide damage is illustrated in Figure 3. Five traces from the somatic and dendritic fields were computer averaged. Stimulus intensity was set to a level that produced approximately half-maximal amplitude of the population spike in the control recordings. It is clear that both the population spike (somatic) and the population synaptic potential (dendritic) are little changed by the presence of peroxide. This is in marked contrast to the decreases that occur in both responses in peroxide alone (see Fig. 5B and Pellmar, 1986).

A synopsis of the protective actions of the agents tested is shown in Figure 4. Differences between two

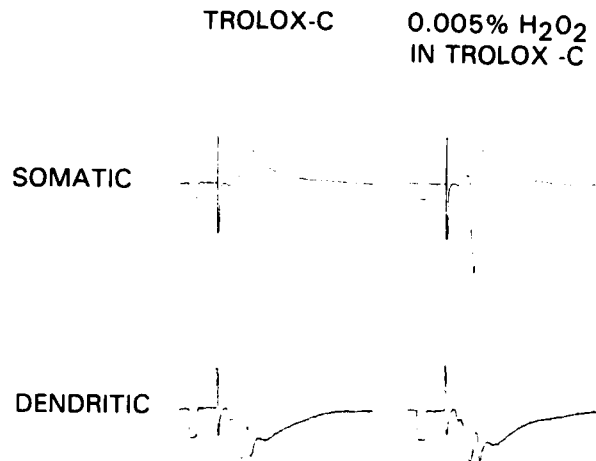


Fig. 3. Electrophysiological records (average of five traces) from stratum pyramidale (somatic) and from stratum radiatum (dendritic) of the CA1 region of hippocampus. Following treatment with 100 μ M Trolox-C 0.005% peroxide had minimal effect on both the dendritic response (the population PSP) and the somatic response (population spike). Calibration pulse: 1 mV, 2 ms.

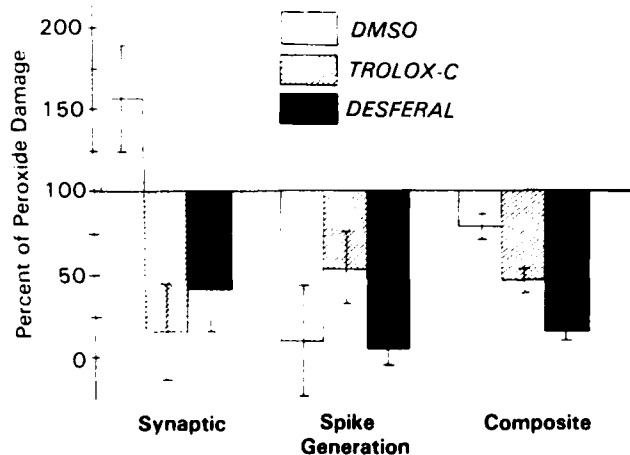


Fig. 4. Relative effects of DMSO, Trolox-C, and deferoxamine on the peroxide-induced decrease in the Population PSP (SYNAPTIC), reduction in the ability to generate a postsynaptic spike (SPIKE GENERATION), and the decreased orthodromic activation of a population spike (COMPOSITE), which combines both synaptic and spike generating deficits. Damage by peroxide in the absence of any protectant is considered to be 100%. Lower values suggest that there is less damage than with peroxide alone while higher values suggest a greater degree of damage. All three protectants were effective on the composite damage and on spike generation. DMSO was not protective against peroxide damage on the synaptic response.

computer-fitted curves can be estimated by comparing the ratio of parameters describing the curves (maximum y value/ x value at half-maximal y value) before and

during exposure to peroxide, as described previously (Tolliver and Pellmar, 1987). The change in the curves in the absence of any protectant was set to 100%; the change in the curves in the presence of protectant would be less than that if the peroxide-induced damage was reduced. It can be clearly seen in Figure 4 that all three of the protectants reduce the composite damage produced by peroxide. Deferoxamine was the most effective, allowing only 16% of the damage produced by peroxide alone. DMSO was the least effective, allowing 79% of the unprotected damage. The relative ineffectiveness of DMSO results from its inability to protect at the synaptic site. If anything, the synaptic damage was greater in the presence of DMSO than in its absence, although this was not statistically significant. All three agents improved spike generation damage from peroxide. Trolox-C was least effective. In Trolox-C, peroxide damage was 54% of the unprotected damage while in DMSO and deferoxamine damage was only 5–10% of peroxide control.

Neither deferoxamine, Trolox-C nor DMSO affected the electrophysiological responses in the absence of peroxide (data not shown). In contrast, thiourea did have direct effects on the orthodromically evoked responses. This prevented a comprehensive study of thiourea using input-output curves. Instead, the amplitude of the half-maximal response was monitored for 30 min in thiourea followed by another 30 min in thiourea plus peroxide.

Thiourea (0.5 mM) also afforded some protection from peroxide damage although exposure to this dose of the scavenger caused the population spike to gradually increase with time (Fig. 5A) ($n = 12$). If an estimate is made by extrapolation of the size the population spike would be 30 min after initiation of application of peroxide, one can observe that the decrement with hydrogen peroxide is less than that seen without the scavenger ($n = 10$). Since the protection was incomplete, 5 mM thiourea was tested ($n = 6$). This dose, however, directly decreased the population spike and was not tested in combination with peroxide. Figure 5B illustrates sample traces of the population spike (average of four) with and without thiourea. In the presence of thiourea (0.5 mM) the amplitude of the population spike is about the same 30 min after exposure to peroxide as it was in the control period. This is somewhat misleading since thiourea was, by itself, increasing the population spike amplitude. In contrast, however, peroxide in the absence of any protectant caused a significant reduction in the population spike ($n = 10$).

Protectants on Lipid Peroxidation

Peroxide has been hypothesized to exert its action on hippocampal neurons, at least in part, through peroxidation of the cell's lipid membranes (Pellmar, 1986,

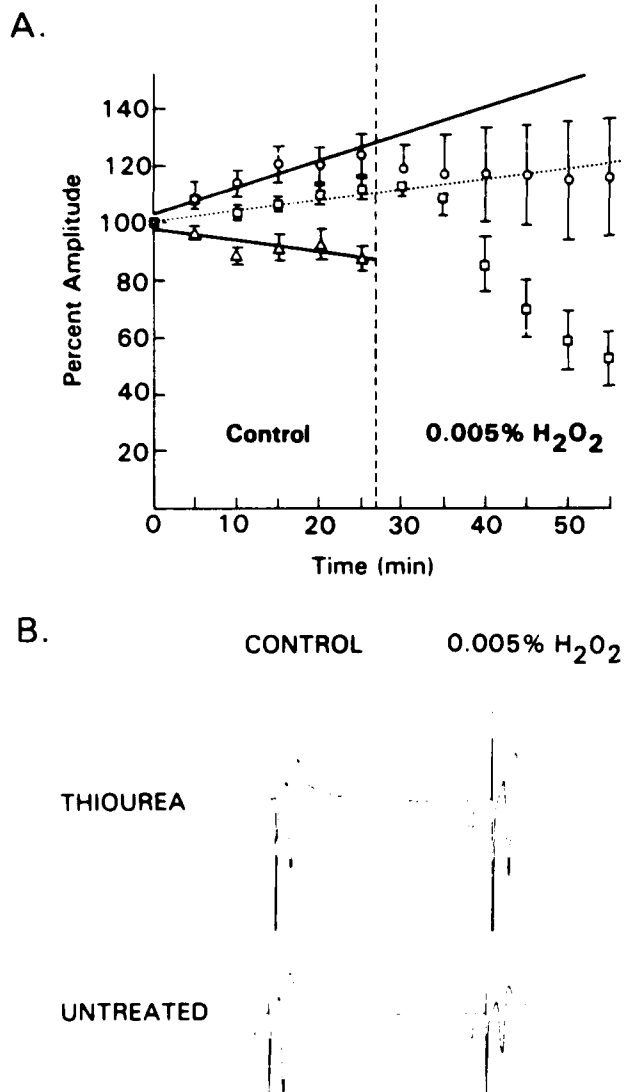


Fig. 5. Thiourea at a dose of 0.5 mM provided some protection from peroxide damage. Orthodromic pathway in CA1 region of hippocampus was stimulated at a frequency of 0.5 Hz, using a stimulus strength that evoked a half-maximal amplitude population spike. Population spike was recorded in stratum pyramidale. Four traces were averaged every 5 min throughout the experiment. A. The amplitude relative to the size of the response at time zero is plotted here. Data were collected for 30 min either with thiourea at the doses indicated (circles and triangles, solid lines) or in the absence of any protectant (squares, dotted line). Peroxide (0.005%) was added at the time point indicated by the vertical dashed line. 0.5 mM thiourea (circles) caused a gradual increase in the amplitude of the population spike while higher doses such as 5 mM (triangles) caused a gradual decrease in the amplitude of the population spike. The reduction in the population spike produced by peroxide in the absence of thiourea was greater than the decrease seen in the presence of 0.5 mM thiourea even when the gradual growth of the response is taken into account (extrapolated lines). B. Sample electrophysiological records. The population spike was minimally affected when 0.005% peroxide was applied in the presence of thiourea but was substantially decreased when thiourea was not present (untreated). Calibration pulse: 1 mV, 2 ms.

DISCUSSION

The data indicate that free radical scavengers can provide protection from peroxidative damage. Both deferoxamine, the iron chelator, and the antioxidant Trolox-C were effective electrophysiologically at the synaptic and spike generating sites and prevented lipid peroxidation. The hydroxyl radical scavenger DMSO effectively prevented both the reduction in spike generation and the increased lipid peroxidation but not the synaptic damage. While the free radical scavenger thiourea was protective, its effects were difficult to interpret due to its direct actions.

Deferoxamine is a very effective iron chelator that can make the metal completely inaccessible for Fenton chemistry (Graf et al., 1984). The nearly complete protection from peroxide damage with deferoxamine strongly suggests iron involvement. This implies that the Fenton reaction is required and therefore it is reasonable to postulate that hydroxyl radicals are mediating the damage. The source of the iron is unclear. While no iron was added to the solution, it is a common contaminant of commercially available compounds [Halliwell and Gutteridge, 1985; Wong et al., 1981]. Free cellular iron is likely to be present in negligible concentrations, but iron chelates of citrate and ATP are available for reaction with peroxide. Several iron binding proteins are also likely to be present in the tissue: hemoglobin and transferrin from residual blood and ferritin intracellularly. These proteins can, under certain conditions, release

1987]. Using the thiobarbituric acid test for MDA, we found evidence for lipid peroxidation with 0.01% hydrogen peroxide (Table I). Resting level of MDA was 119.43 ± 6.66 nmoles/mg wet weight. Following a 30 min incubation with peroxide the levels increased to 145.04 ± 7.81 nmoles/mg. The actions of DMSO, Trolox-C, and deferoxamine were evaluated on peroxide-induced lipid peroxidation. Tissue was pretreated with protectants for 30 min followed by a 30 min exposure to peroxide. As a control, tissue was also exposed to the protectants for 1 hr to evaluate any direct effects of the protectant on the tissue. Deferoxamine and Trolox-C were capable of preventing the peroxide-induced change in MDA without causing any direct effects (Table I). DMSO also effectively prevented any peroxide-induced changes but itself increased the measured MDA.

TABLE I. Effect of Protectants on Malonaldehyde Levels in Hippocampus^a

Treatment	N	Untreated	Drug alone	Drug + peroxide
Control	12	119.43 ± 6.66		145.04 ± 7.81*
Desferal	10	127.69 ± 5.36	129.51 ± 9.47	121.93 ± 8.94
Trolox-C	10	118.46 ± 5.34	119.18 ± 5.83	99.72 ± 6.59
DMSO	10	129.12 ± 4.87	145.02 ± 5.43*	139.54 ± 3.17*

Malonaldehyde was measured by the thiobarbituric acid test and standardized by tissue wet weight (nmoles MDA/mg wet weight). Values are the average of N preparations ± SEM. Slices from one animal were divided into three groups: untreated incubated for 1 hr (untreated), treated with the test drug alone for 1 hr (drug alone), and treated with the test drug for 30 min followed by treatment with the test drug and 0.01% peroxide for 30 min (drug + peroxide).

*Statistically significant difference from paired, untreated tissue; paired t test, $P < 0.05$.

their iron and promote free radical reactions (Halliwell and Gutteridge, 1984, 1985; Aust, 1988).

Although DMSO is an effective hydroxyl radical scavenger (Reuvers et al., 1973; Chapman et al., 1973; Littlefield et al., 1988), it only protected spike generation but not synaptic potentials from peroxide damage. Yet the results with desferal suggest that hydroxyl radicals are involved in both mechanisms. Since hydroxyl radicals are so reactive, they do not diffuse far. Therefore, interaction of peroxide with iron is likely to cause damage near the metal binding site. Although distribution of DMSO may be fairly uniform throughout the cell, hydroxyl radical production is more likely to be site specific (Chevion, 1988). As a consequence, DMSO might be available in a sufficient concentration to scavenge all the hydroxyl radicals at one site while at another site, the concentration may be inadequate. In addition, steric limitations may prevent access by the scavenger more at one location than another (Chevion, 1988). A differential effect of DMSO on synaptic and spike generating mechanisms therefore may be the result of the nonuniform distribution of iron.

Trolox-C, a vitamin E analog, prevented both the decrease in the synaptic potential and the generation of action potentials caused by peroxide. It is well established that vitamin E scavenges the peroxy radical in the lipid membrane that occurs with the initiation of lipid peroxidation (Doba et al., 1985; Halliwell and Gutteridge, 1985; Burton et al., 1985; Niki, 1987). Doba et al. (1985) suggest that Trolox-C, despite its water solubility, is sufficiently lipophilic to penetrate and traverse lipid bilayers and to scavenge the lipid soluble peroxy radical. Other mechanisms of action for Vitamin E have also been hypothesized. For example, Pascoe et al. (1987) suggested that Vitamin E is effective in chemical toxicity by maintaining levels of cellular protein thiols such as glutathione. It is possible that Trolox-C provides protection against impaired spike generation and synaptic damage through two distinct mechanisms.

Peroxide impairs spike generation and synaptic transmission through a free radical mechanism. The ac-

tions of the iron chelator deferoxamine suggest that hydroxyl radicals are likely to be the initiator for both sites of damage. Yet the mechanisms of action of the radicals at the two sites are likely to be distinct. ⁶⁰Cobalt radiation, like peroxide, produces both spike generation and synaptic damage (Tolliver and Pellmar, 1987). Spike generation damage did not show dose-rate dependence which is consistent with a lipid peroxidation mechanism. The observation that DMSO prevents impairment of spike generation and lipid peroxidation, but not synaptic damage, strengthens this correlation. Synaptic damage from gamma radiation was sensitive to dose-rate suggesting a distinct mechanism. Synaptic damage from peroxide and radiation might be due to an oxidation of membrane proteins. Protein oxidizing agents, chloramine-T and n-chlorosuccinimide, produced only synaptic damage, and did not impair the spike generation (Pellmar and Neel, 1989) nor induce lipid peroxidation (Pellmar and Lee, unpublished data). In conclusion, the data suggest that peroxide reduces synaptic potentials and impairs postsynaptic generation of action potentials through two distinct free radical mechanisms.

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